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Antibacterial efficacy of a series of biologic dressings in a septic burn wound model

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ABSTRACT

This study was undertaken to assess the efficacy of both fresh and preserved xenogeneic amniotic membrane as biologic dressings in reducing log bacterial counts of infected burn wounds in comparison with fresh allogeneic skin.

Materials and Methods

Sixty-four Sprague-Dawley rats (300 gm) were anesthetized and subjected to standard 20% BSA dorsal scald burns (Day 0). All wounds were immediately surface inoculated with 10^8 P. aeruginosa from 18 hour broth cultures. At Day 5, the 60 survivors underwent escharectomy and random biopsies of the sub-eschar wounds. Each wound had bacterial counts $>10^5$ organisms/gm tissue. Animals were then randomized into four treatment groups: 1) Control - dry sterile gauze dressing; 2) fresh split thickness skin allograft; 3) freshly harvested, sterile human amniotic membrane; 4) lyophilized sterile human amniotic membrane (obtained fresh, freeze-dried to moisture content of < 0.05%). Before application, lyophilized amniotic membrane was reconstituted by immersion in buffered Ringer's lactate, pH 7.4. All amniotic membrane was applied chorion-side to the wound. All dressings were changed at Day 7 (52 survivors) and removed on Day 9 (45 survivors). On Day 7 and Day 9, 4 randomized wound biopsies were taken from each animal.

Results and Conclusions

Treatments with fresh and preserved xenogeneic amniotic membranes did not differ significantly from one another or the control group. Neither treatment decreased log bacterial counts. Fresh allogeneic skin significantly reduced log bacterial counts at Day 7 ($p<0.05$) and at Day 9 ($p<0.001$). These results are consistant with the absence of any intrinsic antibacterial properties of either fresh or preserved amniotic membrane. The observation that fresh allogeneic skin is effective in reducing bacterial counts in this model, supports the hypothesis that an initial attempt at "take" may be required for antibacterial efficacy of a biologic dressing.

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INTRODUCTION

Biologic dressings are useful in the treatment of partial and full thickness skin loss to functionally replace the skin until such time as it can be restored either through regeneration or autografting. Material, usually skin, derived from many animal species has been used. Currently cadaver and porcine skin are the most popular and are commercially available, but at a relatively high cost. Amniotic membrane has recently enjoyed new popularity as a biologic dressing as is evidenced by numerous clinical reports. It has been postulated that fresh amniotic membrane is capable of reducing the quantitative bacterial counts both in clinical (human) and experimental (animal) burn wounds. This project was designed to further investigate this hypothesis and explore the possibility of lyophilization as a method of preservation of the amniotic membrane while retaining its antibacterial effects. Preservation with retention of antibacterial efficacy would allow amniotic membrane to be used in areas where it has been previously unavailable. The following review of the literature will summarize the use of biologic dressings with emphasis on amniotic membrane, concentrating on its recent clinical revival, methods of preservation, and investigations bearing on the mechanisms of its antibacterial effect. In this discussion the term "amniotic membrane" will refer to the placental membranes in the natural state, "amnion" to amniotic membrane stripped of chorion, and "chorion" to amniotic membrane

stripped of amnion. Terms in the older literature such as homograft and heterograft, have been replaced with the currently accepted terms allograft and xenograft, respectively.

A.Biologic Dressings

Biologic dressings may be used to promote healing of partial thickness wounds. They are however especially useful and possibly lifesaving when re-establishment of permanent skin continuity by autografting is temporarily prevented because donor sites are lacking, the patient is too ill to withstand the graft harvesting procedure, or the recipient site is not yet ready. In this case the biologic dressings have the capacity to 1) temporize - allowing multiple same-site harvesting of split-thickness skin graft or improvement in the general status of the patient and 2) prepare the recipient site for grafting. Adequate supplies are available because allograft and xenograft material may be used.[1-5] Permanent "take" of the biologic dressing is not desirable and is prevented by frequent dressing changes, that is, every 48-72 hours or more often if treatment of infection is also required. Prevention of "take" by dressing changes avoids the morbidity of a debilitating rejection episode. The frequent changes encourage debridement of necrotic or infected tissue.

It has been demonstrated that for consistent autograft survival quantitative bacterial counts in the recipient bed must be less than 10^5 /gram of tissue.[6] Thus the ability of biologic dressings to reduce or prevent infections has been of great interest. This will be discussed in more detail below.

The early history of biologic dressings, as well as the advantages and disadvantages of their use, has been well summarized [7-8] and will not be discussed here. It should be noted that much effort has been expended to develop a synthetic material with characteristics such as adhesiveness, porosity, and bacteriostasis, required for an artificial skin, with limited success.[9-14]

B. Amniotic Membrane

The use of amniotic membrane as a graft was apparently first suggested by Thornton, at the time a senior medical student at Johns Hopkins Hospital, and reported by John Davis in 1910 in his monumental review of skin transplantation at the same institution.[15] Not specifying any of the technical details of his experiments, Davis notes that the grafts were not successful but suggests that the "material is well worth a trial." Early investigators believed that this human embryonic material was less likely to be rejected than animal skin and would provide the necessary ingredients for regenerating skin, being derived from ectoderm and being an "extention of the fetal skin."[16]

In 1913 Sabella[16] and Stern[17] published independent reports of their successful use of amnion in the treatment of "burns, scalds, varicose ulcers, and denudations of traumatic origin." It is apparent that they believed there was actual take and incorporation of the fetal cells into the new skin. Stern notes that a major advantage in the use of amniotic membrane is the avoidance of anesthesia and the second wound neccessary at the donor site for autografts, a point which is still important today.

Amniotic membrane then became popular for a time in neurologic and opthalmologic surgery, but further discussion of its use is generally absent from the plastic surgery literature until the end of World War

II, which generated large numbers of burn casualties and the spectre of thermonuclear war. Papers published in the early 1950's discuss the neccessity of developing and stockpiling substitutes for homograft skin, the supplies of which would be quickly exhausted by an atomic war.[18]

The next stage in the development of use of amniotic membrane was marked by independent reports of its clinical use in a variety of situations. Workers described unique methods of preparation or postulated mechanisms for its unusual properties. Unfortunately there is much confusion of terms, lack of precision in description of methods used, and most clinical and experimental reports are of a subjective, qualitative nature. An attempt will be made to summarize those findings which are relevent to the current understanding of the role of amniotic membrane, and its limitations.

Troensgaard-Hansen used boiled amnion, placing the epithelial surface against the wound, as a graft in the treatment of chronic skin ulcers in elderly bed-ridden patients and reported complete healing.[19] He recommended 10 weeks of bed rest during which the dressing was not disturbed. A student of his, Kidd, dried the boiled amnion over cotton wool balls and took a supply with him on an Arctic cruise.[20] After 7 months of storage the amnion was rehydrated by boiling in saline and used to cover a 6 cm^2 full thickness wound. Kidd states that at the 7-day dressing change the wound was completely re-epithelialized.

Sterling reported his use of amniotic membrane in the pre-autograft treatment of debrided human flame wounds.[21] Van Duyn emphasized the

importance of an appropriately timed escharectomy in the treatment of third degree burns even if permanent skin coverage was not yet available.[22] He recommended use of amniotic membrane to prevent the loss of protein, fluid and electrolytes from open, weeping, granulation tissue, while awaiting autograft skin.

Dino et al. were impressed with the utility of amnion in the treatment of second degree burns and split-thickness skin graft donor sites.[23] They emphasized the rapid relief of pain after the application of the dressing. Histologic study of biopsies of these donor sites failed to show vascularization of the amnion. The membrane was, however, "richly invaded with phagocytic cells." Dino and coworkers later established an amnion bank, after demonstrating that bacterial growth was prevented when amnion was stored in solutions of sodium hypochlorite, saline with penicillin and streptomycin, or saline with kanamycin at 5°C or up to 14 days.[24] Pigeon stored amnion in antibiotic solution for up to nine months and used this this amnion to successfully treat second degree burns.[25] He felt that the viability of the tissue was not a prerequisite for good outcome, recognizing there was no evidence that the amnion cells reproduced on the skin.

Trelford et al. used allogeneic amnion (amniotic membranes stripped of chorion) as a dressing on the perineum following radical vulvectomy by suturing it into lace mesenchymal-side place mesenchymal side-down (amnion epithelium up). They stated that unpublished preliminary studies indicated a lack of "cellular immunologic response" when the amnion was used in this manner. They attributed their successful

clinical results to prevention of this reponse which was accomplished by 1) avoidance of the "antigenic chorion" and 2) application of the mesenchymal side to the wound. In their unpublished pilot studies application of the amnion mesenchymal-side down did not lead to capillary and cellular invasion of the amnion in a xenogeneic model.[26-28]

Douglas demonstrated histologic survival of fresh xenogeneic amnion or chorion when grafted onto experimental dog wounds. However she followed the wounds for only 17 days.[29] In light of this she treated a burn wound with amnion allograft and two split thickness skin graft donor sites with chorion allograft and again demonstrated histologic survival of the cells of both membranes for about three weeks.

Later Douglas et al. using the transparent tissue chamber technique, studied the behavior of human amnion grafted onto full thickness mouse wounds.[30] They first noted thrombosis of vessels in the recipient bed, followed by neovascularization of this bed. At no time was penetration of the chorion graft by the neovascular process observed. However, the graft remained viable for an average of 12 days as compared to an average viability of 5.6 days for an allograft skin control (thickness not specified). Douglas et al. also studied amniotic membrane autografts in the same model. Significantly they demonstrated no recipient bed vessel thrombosis. Rapid re-epithelialization of the wound surface by cells originating from the wound edges occured. Whether these cells were of dermal or placental origin is not clear.

Colocho et al. studied the question of neovascularization of amnion.[31] Both fresh and viable-preserved (frozen in 20% glycerin; viability asesed post-thaw by eosin excusion) amnion were used in clinical (65 split thickness skin graft donor sites and 42 partial thickness burns) and experimental (applied to open wound on the rat panniculus carnosis or buried subcutaneously) models. Careful studies demonstrated the absence of any vascular connections between amnion graft and any of the beds in both clinical studies (allogeneic) and experimental models (xenogeneic).

More recently the results of large clinical trials of amniotic membrane have been reported; most have been anecdotal. Chuntrasakul described his subjective experience with 265 patiets, including 215 burn patients, using fresh allogeneic amniotic membrane.[32] 140 of the patients had "minor" burns - mostly partial thickness, 2-15% BSA - and were treated by 1-3 applications of amniotic membrane amnion-side down. Eighty per cent remained free of infection by clinical signs and none required hospitalization. Seventy-five of the burns were classified as "major" - mostly full thickness, 13-75% BSA. These wounds were treated by application of the amniotic membrane chorion-side down after escharectomy (dressings changed every 48 hours). The membranes were reported to have decreased fluid, electrolyte, and protein loss, and increased vascularity of the granulating wound. However, no data was provided. Eldad and coworkers reported a series of 30 patients (15 burns) in whom allogeneic amniotic membrane was used.[33] In partial thickness skin loss the amnion side was applied to the wound while in

full thickness loss the chorion side was applied against the wound with the specific aim of enhancing granulation tissue formation on denuded areas as a prelude to skin graft application. Walker, Cooney, and Allen compared fresh allogeneic amnion to Furacin impregnated gauze in 110 children presenting with second and third degree burns.[34] In the 58 patients requiring hospitalization because of size, depth, or location of wound, they demonstrated that those treated with amnion (18, av burn 13% BSA) required hospitalization periods of significantly shorter duration (16 vs 27 days) than those treated with the Furacin gauze (40, av burn 12% BSA). However the time required for healing was not significantly different. Bose has reported in detail his management protocol for burn wounds where depth of injury is not known or is variable, using fresh allogeneic amniotic membrane in 15 patients.[35]

Thompson and Parks reported storage of human amniotic membrane in 10% glycerol at -80 C for periods as long as 300 days.[36] The material was then used as a biologic dressing in the interim management of pediatric burn wounds. Best results were seen when the membrane was used as a cover dressing over meshed skin autograft on fascial surfaces. Rapid closure of the graft interstices with graft loss of less than 5%, was observed.

Finally Gruss and Jirsch used fresh allogenic amniotic membrane applied chorion-side down in 120 patients with full thickness defects, including ulcers, elective, infected or contaminated surgical wounds, saucerized bone, burns, and traumatic soft tissue wounds.[37] Twenty-three of these wounds were clinically infected. The membranes were used to

reduce the log bacterial count. "Take" of the membranes was used as a clinical indicator of reduction of bacterial counts to below 10^5 bacteria/gram of tissue. They defined "take" clinically, i.e. when firm adherance of the membrane to the wound bed without underlying accumulation of pus or debris was observed. When "take" was evident, delayed wound closure was accomplished. Gruss and Jirsch concluded that the "take" of human (allogeneic) amniotic membrane applied to the wound surface was invariably a good predictive index of successful autograft take or wound closure.

Many clinicians have observed and reported alterations in wound healing - rapid re-epithelialization, enhanced granulation tissue formation, relief of pain - when amnion or amniotic membrane is used as a wound dressing. Recent studies have attempted to elucidate the mechanism(s) whereby amnion exerts these reported beneficial effects. One such effect is an apparent acceleration of wound healing. Babat and Kothary studied, by the transparent chamber technique, rabbit amnion grafted onto an excisional wound on a rabbit ear, over a 20 day course.[38] Membranes were collected by Ceasarian section, maintained in Eagles MEM with added antibiotics, and were used within 10 days. They observed an earlier appearance and faster progression of granulation tissue as compared to their wound controls. In addition, grafted wounds were characterized by a greater degree of fibroblast and epithelial cell migration, increased collagen synthesis, and more rapid growth of a well-formed epithelial layer. However gross observation revealed no difference between the grafted and the control wounds. Specifically,

there was no enhancement of wound angiogenisis by the amnion grafts. Based on these observations, Babat and Kothary concluded that wound healing was accelerated in the presence of allogeneic amnion grafts. They speculated that a mechanism existed whereby living amnion promoted "tissue differentiation." It should be noted that in this study amnion was maintained in tissue culture medium prior to use, and this method of storage may have affected its properties. Additionally, rabbits were treated with immunosuppressive doses of cortisone acetate - doses known to affect wound healing.[39] Importantly, in this allograft model, there was no evidence for vascular penetration of the amnion by the host bed.

In contrast to the experimental study above demonstrating no apparent effect of amnion on angiogenisis, Faulk and co-workers reported clinical trials with amnion in which they suggest it is endowed with "angiogenic factors" similar to the vessel growth promoting factors said to be produced by tumor cells. Faulk *et al.* using histologic and immunohistologic techniques (antiserum to Factor VIII as a marker of endothelial cells) studied 15 human chronic leg ulcers *via* biopsies taken prior to and 5 days after application of human amnion dressings. In these experiments human amniotic membrane was maintained in culture for up to three weeks; chorion was stripped off immediately before application. H & E, reticulin, and immunoflourescent stains demonstrated an increase in the number of capillaries in the ulcer bed, along with increased patency of vesseels, thinned connective tissue, and increased numbers and brilliance of the immunoflourescent granules. Thus, in this allogeneic model there was evidence of increased

vascularity after exposure to amnion.[40-41]

C. PRESERVATION OF AMNIOTIC MEMBRANE

One of the attractive features of amniotic membrane as a biologic dressing is its ready availability in great quantities, especially in the large hospitals where it is likely to be needed. However widespread use of amniotic membrane has been limited by the high manpower costs of preparation and the difficulties encountered in storage of fresh, sterile material. Many investigators have attempted to solve the problem of storage. Basically their solutions can be divided into two classes - those which maintain viabiity of the amniotic membrane and those which do not. Methods of preparation have included serial washes in physiologic solutions,[41] immersion and storage in various bactericidal agents,[24] storage at low temperature,[34] freezing,[31] drying,[20] boiling,[18] and storage in tissue culture.[42] Some of these techniques have been discussed above. One question which has never been answered, however, is the question of whether amniotic membrane must be living for it to be used successfully as a biologic dressing.

Rao and Chandrasekharem recently suggested a possible solution to the twin problems of preservation and provision of adequate supplies by exploring the use of dried bovine amnion.[43] They air-dried both human and bovine amnion after removing surface contaminents with a dilute hypochlorite rinse. The dried amnion was then sealed in envelopes and radiation sterilized. The dried amnion (human or bovine) was stored for up to nine months before use. Safety, durability, bacteriostatic

activity, effectiveness, and adherence of the dried sterile bovine amnion was first evaluated in a xenogeneic (rabbit) burn wound model. Results were reported as "satisfactory" but noted specifically were sterile bacterial cultures from the amnion treated wound area while bacterial cultures from the control area were positive for Staphylococcus and Pseudomonas. A clinical trial in 70 patients (58 burns)was then undertaken. Of the 50 burn patients for which information is available, 43 (86%) had burns of less than 30% BSA. Twenty-seven (62%) of these were superficial burns. In the clinical study both fresh and dried allogeneic (human) amnion were compared with dried xenogeneic (bovine) amnion. The amnion was applied over both superficial and deep burns after minimal debridement immediately after admission. In the treatment of deep burns amnion was reapplied post-escharectomy and when there were signs of infection or autolysis of the amnion. The authors state that there were no differences between the various types of amnion used. However, how this was established is not evident from the paper; only subjective observations were reported.

Preservation of skin (human or porcine) by lyophilization (freeze-drying) for use as a temporary biologic dressing is a common practice.[44-45] It is important to note that the lyophilization process does not preserve tissue viability and therefore permanent "take" is precluded. Only two studies could be found in the literature in which lyophilization was used to preserve amniotic membranes. In the first study, Klen described a rather complicated and time consuming method used at the Tissue Bank Faculty Hospital in the CSSR.[46-47] Strips of

amnion, chorion, or amniotic membrane, were placed on a plastic net, rolled, and stored under a nitrogen atmosphere in a "deep freeze" prior to lyophilization. Materials were then lyophilized for a period of 48 hours. Dry sterile nitrogen was then admitted to the jars and they were sealed. The lyophilized membrane was arbitrarily assigned a shelf life of one year. Rehydration was accomplished of the membrane for 10 minutes in sterile water (not physiologic solution) containing antibiotics and calcium gluconate. Klen stated that membranes prepared within two hours of delivery and stored for less than one week before lyophilization provided the more "successful" grafts.

In a procedure described more recently by Notea et al., amniotic membrane was spread on gauze covered tray and frozen at -20 °C.[48] The frozen amniotic membrane was lyophilized for 24 hours, and placed in polyethylene sacks, and sterilized with ethylene oxide. Amniotic membranes prepared in this manner were stored at room temperature. They were rehydrated by immersion in physiologic saline solution. The authors used reconstituted amniotic membranes in six cases of various full thickness skin injuries. They concluded that the freeze-dried amniotic membrane compared favorably with fresh amniotic membrane with respect to its effects (adherence, enhanced epithelialization, healing time) on the wound bed. However their report did not document this by any objective criteria.

The studies of Klen and Notea constitute the only reported investigation of the use of a lyophilized amniotic membrane as a biologic dressing in either allogeneic or xenogeneic wounds. No studies

detailing the antibacterial efficacy of lyophilized amniotic membrane as compared to fresh material have been reported to date.

Recently, it has been shown that lyophilization of bone, skin and certain cultured cells changes the cell surface lipoprotein histocompatibility antigens, rendering cells and tissues only weakly stimulating of cellular immunity after transplantation in allogeneic models.[49] while this observation has been confined to tissues other than amniotic membrane, there is no reason to suspect that amniotic membrane would behave any differently. Thus, if the antibacterial properties of the membrane are preserved after lyophilization, a concommitant decrease in antigenic potential could only be to the benefit of the allo- or xenogeneic wound in which it might be used.

D. ANTIBACTERIAL PROPERTIES OF AMNIOTIC MEMBRANE

Eade noted in 1958 that despite the presence of bacteria in granulation tissue, split thickness autografts took well.[50] He theorized that this occured because of a rapid removal of bacteria. In his clinical experiments, he observed that a reduction in quantitative bacterial levels occured by 24 hours post-grafting in most patients undergoing either autografting or allografting of either fresh or lyophilized skin.

Since that time the ability of either autograft or allograft skin to reduce wound bacterial levels has been repeatedly confirmed. In fact, the ability to decrease bacterial levels is considered an important criterion in the selection of a biologic dressing. Biologic dressings endowed with this property can be used both therapeutically to "clean-up" wounds prior to grafting and prophylactically to suppress bacterial overgrowth in wounds which for other reasons cannot be grafted.

Robson and Krizek undertook a quantitative study of the antibacterial efficacy of amniotic membrane in an infected xenogeneic wound model.[51] Their reported results have become a standard for comparison, and it is therefore important to review their study in detail. The animal was a 250 gram Sprague-Dawley rat subjected to a 20% full thickness scald burn. Immediately after burning, the burned areas were topically inoculated with $1x10^8$ Pseudomonas aeruginosa from an 18 hour broth

culture. Animals surviving at Day 5 post-burn (38/50) underwent escharectomy. Bacterial infection was confirmed by quantitative tissue cultures on the day of escharectomy. Each wound was divided into three equal segments along the rostral caudal axis. To each segment of each animal was applied one of three dressings: fresh human amniotic membrane, split thickness human skin, dry sterile gauze dressing (control). Wound biopsies for quantitative log bacterial counts were performed at 48 hours post-escharectomy, at which time appropriate fresh dressing were applied. Biopsies were repeated at 96 hours post-escharectomy. Results at 96 hours post-escharectomy were reported to be most significant. The authors reported a reduction in log bacterial counts in 100% of the amniotic membrane treated wounds, 90% of skin-grafted wounds, but in only 40% of control wounds. Of the remaining control wounds, 2/3 showed no change in bacterial count while 1/3 exhibited increased log bacterial counts. The geometric mean of the bacterial counts from the group treated with human split thickness skin was 10^6 bacteria/gram, compared to 10^3 bacteria/gram in the group treated with amniotic membrane. While the authors report signifigance of p values, they fail to report the number of surviving animals in any group at any time post-escharectomy, i.e. from Day 5 when the first dressings were applied to the termination of the study on Day 9. Additionally they do not report what they considered to be a "significant reduction" in bacterial count.

Robson and Krizek next studied the question of whether human amniotic membrane or human skin exhibited any intrinsic antibacterial properties

in vitro. [53] To an inoculum of 10^3 to 10^8 P. aeruginosa or E. coli in an unreported volume of thioglycolate medium, was added 50-200 mg of either homogenized human skin or homogenized human amniotic membrane. Within 24 hours after addition, all cultures exhibited counts of 10^8 bacteria/ml.

Based on these experiments the authors concluded that neither human amniotic membrane nor human skin posess inherent bactericidal or bacterostatic properties. The study is subject to criticism, however in that the methodology chosen really is not adequate to discern any postulated intrinsic antibacterial factors. Furthermore the concentrations of the initial inoculum and the homogenate are not reported, and the results are difficult, if not impossible to interpret. There is ample evidence to suggest that these factors may affect the growth curve rather than the final concentration of bacteria. Early and serial sampling should be done to detect this.

Later, Robson *et al*. developed a model to study the effect of dressings on the growth of bacteria, hypothesizing that a low initial inoculum would more closely simulate the clinical situation of an infected granulating wound bed.[52] Four full thickness defects (1.5 cm^2) were made on the backs of each of 20 rats. Each defect was immediately innoculated with $5x10^5$ P. aeruginosa and covered with one of 5 dressings: split thickness autograft skin, split thickness allograft skin, split thickness xenograft porcine skin, split thickness xenograft human skin, or fresh xenogeneic human amniotic membrane. At the end of 48-72 hours, quantitative tissue biopsies revealed 10^6 bacteria/gram in

the allograft and xenograft skin treated wound beds as compared with 10^3 bacteria/gram in the xenogeneic amniotic membrane or autograft skin treated wounds. The authors concluded that amniotic membranes were superior to allograft and xenograft skin in decreasing wound bacterial counts. This second model is also open to criticism, as they presented no evidence that equivalent infection levels were achieved beneath the dressings, which were applied immediately after wound innoculation. The dressings may have affected the bacteria before their invasion of the wound bed. Thus, their experimental model does not simulate the clinical use of the dressings.

Although these studies have become the standard of reference with respect to the antibacterial efficacy of amniotic membrane as compared with other biologic dressings, critical experimental details were unfortunately omitted by the authors: the description of burning is too sketchy and refers the reader to other works which are no more helpful, the same strain of Pseudomonas was not used throughout any series of studies, authors failed to note whether amniotic membrane was applied amnion-side down or chorion side down. Probably most importantly, the authors utilized 3 different dressings in one wound. The small areas involved and failure to isolate one from another produce cross-contamination and sampling errors which raise serious doubt as to the validity of the quantitative bacterial counts. Finally, as noted earlier information presented in the studies is incomplete and thereby does not permit proper statistical analysis.

Walker, Cooney and Allen demonstrated in a clinical trial that amnion was better than Furacin gauze in limiting the number of positive bacterial surface cultures of several common pathogens (cultured during dressing change).[34] Indeed, in their trial 18% of burn wounds demonstrated repeated sterile cultures as compared with 100% contamination of the Furacin treated wounds.

Salisbury et al. have compared the bacterial clearing effects of amnion (actual membrane not specified), homograft skin and porcine xenograft in a clinical trial of second and third degree burn wounds in 16 patients.[53] Most wounds exhibited no change in the log bacterial count at 24 hours. However they limited use of the biologic dressings to those wounds judged clinically not to be ready for grafting and evaluated the wounds only once, 24 hours after application of the dressing. In addition, the origin and preparation of the "amnion" and the type of allograft used is not specified, making this study difficult to interpret.

Many authors have postulated mechanisms for the apparent antibacterial effects of amniotic membrane.[54] Those who subscribe to the mechanical theory assert that the amniotic membrane achieves an intimate biologic closure of the wound through fibrin-elastin bonding, encouraging a functional circulation in the granulation tissue thereby increasing the turnover of leukocytes. Additionally, changing the membrane frequently serves to debride devitilized tissue. Those adhering to the biochemical theory claim that amniotic membrane is endowed with intrinsic antibacterial factors. The existence of

bactericidal and bacterostatic systems in amniotic fluid has been well documented in vitro.[55-63] Whether these factors are produced by the amniotic membrane and/or are retained after delivery in sufficient quantities to affect wound bacteriology is not known. A third theory, of "take", combines elements Of the biochemical and mechanical theories. Some experimentors hypothesize that "take" of the amniotic membrane is required for antibacterial efficacy. This is usually intended to mean the establishment of a biologic interconnection of the graft and the wound bed by vascular invasion of the amniotic membrane. This is achieved only when the rejection mechanisms are attenuated either because of suppression or allogeneity of the graft. Vascular invasion with active blood flow would achieve beneficial effects by well known mechanisms, as well as distributing the postulated intrinsic antibacterial factors of amniotic membrane.

E. STUDY DESIGN

To summarize, recent clinical and experimental investigations have clarified details of the use of allogeneic amniotic membrane in the treatment of skin wounds.[64] Partial thickness losses are treated by application of the amniotic membrane with the epithelial amnion side in contact with the wound. This discourages vascular penetration and is reported to encourage epithelialization. When applied in this fashion, the amniotic membrane is removed only if there is evidence of infection beneath it. Otherwise healing progresses to completion under cover of the dessicating membrane which falls away after epithelilization is complete. Full thickness losses are treated by debridement of necrotic tissue followed by application of the amniotic membrane chorion-side down. This can be done even in the face of residual wound infection, providing that the amniotic membrane is changed frequently. Apparent "take" of the amniotic membrane to the wound bed as judged by its adherence to same is a good indicator of low bacterial counts in the wound. A biologic dressing probably should not be used in areas of variable depth of injury with potential for infection in order to prevent conversion of the entire wound to a full thickness loss.[65]

Although sufficient clinical experience has been accumulated to permit use of amniotic membrane as detailed above, there remain large gaps in our knowledge. Are there intrinsic factors in amniotic membrane which affect wound healing and wound flora? Must there be vascular

ingrowth for the amniotic membrane to function effectively as a biologic dressing over full thickness defects, i.e. must there be a "take" ? Must the amniotic membrane be viable (consist of living cells) to exert its beneficial effects? Finally, how does the heterogeneity of the biologic dressing affect its ability to function?

The goal of this project is to further investigate the antibacterial effects of amniotic membrane and to explore the possibility of using lyophilization as a method of preservation of the membranes. If the non-viable lyophilized amniotic membrane functions as effectively as the viable fresh amniotic membrane it would imply that the beneficial properties are independent of viability dependent mechanisms such as vascular ingrowth, and support the mechanical or biochemical theories. The parameter chosen for study was the effect of the biologic dressing on quantitative wound bacteriology. Quantitative tissue bacterial counts are simple to perform, reproducible, reliable, and represent a parameter by which clinicians judge the success of a biologic dressing. Because the work of Robson and Krizek has become a standard of reference, frequently cited as demonstrating antibacterial efficacy of amniotic membrane, it was considered important to replicate their xenograft septic burn wound model. Their original design used three dressings on a single wound.[51] As discussed above this does not allow clear separation of the individual dressing's effects. Therefore in this project only one dressing type was used for each wound.

The experimental design was therefore as follows: Rats received a standard 20% dorsal scald burn[66-68] which was immediately surface

inoculated with a pathogenic strain of P. aeruginosa.[69-71] On Day 5 post-burn, escharectomy was performed and quantitative bacterial tissue counts were done to establish wound bed infection. Rats were randomized into four groups, receiving fresh xenogeneic human amniotic membrane, lyophilized xenogeneic (human) amniotic membrane, fresh allogeneic full thickness skin, or plain sterile gauze. The latter two were considered controls. On Day 7 the dressings were removed and biopsies for quantitative tissue bacterial counts were taken. Appropriate new dressings were applied. On Day 9 the dressings were removed, biopsies taken and the rats were sacrificed. Logistical problems prevented accomplishment of certain procedures at one time so that the experiment was performed in two blocks, with starting (burn) days about one month apart.

MATERIALS AND METHODS

A. Animals

Male albino Sprague-Dawley rats (av wt 312 gms) were acclimatized for 1-2 weeks, housed in groups of 10 at the Animal Care Facility, Yale School of Medicine. After burning, rats were placed in individual wire screen "drop-through" cages to minimize cross-contamination and innoculation of the wound by fecal flora, and kept in the Plastic Surgery Laboratory. Rat chow and water were available ad libitum.

B. BACTERIA

A pathogenic strain of Pseudomonas aeruginosa was obtained from the clinical bacteriology laboratories of Yale-New Haven Hospital. This isolate (YNHH-9621-1981) was used to inoculate a trypticase soy broth culture. During log phase of growth, samples were taken and stored in 5% glycerin at -70°C for future use. Before each experiment one of these samples was used to inoculate a new TSB culture.

C. PREPARATION for BURN

Rats were anesthetized for burn and escharectomy using sodium pentobarbital, 30 mg/kg IP as an initial dose, supplemented by 10 mg/kg IP when neccessary. During dressing change and wound biopsy, 10 mg/kg IP sodium pentobarbital was sufficient for sedation.

Weight of rats was recorded. The entire dorsum of the body was then closely shaved using a mechanical clippers.

D. BURN

Rats were subjected to a 20% BSA standard dorsal scald burn using a simple device. A sheet of Plexi-glass (14x28cm) with a window (5x8cm) was used to cover a stainless steel pan (5x12x22cm) filled with water. The pan was set on a metal stand and the water heated to 75 ^{o}C using a Bunsen burner. The shaved dorsum of the rat was placed against the window for 20 seconds. Literature review and preliminary studies indicated that this scald (75^{o}C, 20 secs) caused a full thickness injury. The body surface area of the rat was calculated using the formula $BSA=kW^{2/3}$, where W is the weight in grams and k is a constant (empirically determined to be 11).[72] The window size chosen represents 20% of the body surface of a rat of the average weight.

E. BACTERIAL INNOCULATION

After burning the dorsum of the rat was immediately dried using sterile gauze. After several minutes were allowed to pass for cooling of the burned skin, the surface of the scald was inoculated with P. aeruginosa (1ml of 18hr broth culture) by swabbing. The broth culture was backplated at the time of inoculation to demonstrate a bacterial concentration of $1x10^{8}$ /ml.

F. ESCHARECTOMY

On Day 5 post-burn the eschar was sharply dissected away. Care was taken to leave the underlying panniculus carnosis. Dimensions of the eschar were measured and recorded for each animal before escharectomy.

G. QUANTITATIVE CULTURES

Four random wound bed tissue samples (one from each of the four quadrants) were taken immediately from each rat after escharectomy on Day 5, during the dresssing change on Day 7, and before the rat was sacraficed on Day 9. These four samples were pooled and used to define quantitative bacterial tissue counts for each rat according to a method previously described and in clinical use at Yale-New Haven Hospital.[73-74]

H. TREATMENT PROTOCOL

After escharectomy the rats were randomly assigned to one of three wound treatment groups. Treatments consisted of dressings of 1) fresh human amniotic membrane, 2) lyophilized human amniotic membrane, 3) fresh full thickness allogeneic rat skin. A control group received wound dressings consisting of dry sterile gauze. The dressings were applied as described below immediately after biopsy samples were obtained on Day 5 (after escharectomy),and on Day 7 (after removal of the old dressing).

I. DRESSINGS

1) Amniotic Membrane

Amniotic membrane was aseptically collected by the nursing staff of the Yale-New Haven Hospital Obstetrical Division at delivery from mothers who fit previously established criteria. The membranes were stored individually in sterile containers at 4°C and transferred to the Plastic Surgery Laboratory within 24 hours. Any membranes with obvious meconium staining or foul odor were discarded. Healthy appearing membranes were individually washed under sterile conditions using 5 rinses of 500 ml each of sterile saline. The third rinse contained 0.025% sodium hypochlorite. During rinsing, the membranes were gently agitated by hand to dislodge adherent blood clots. Care was taken not to strip the amnion from the chorion. Biopsies of all membranes were subjected to aerobic bacteriologic cultures (blood and MacConkey agar). The membranes were returned to cold storage in individual sterile containers which were labelled by date of collection. If the bacteriologic cultures were without growth at 36 hours, membranes were considered sterile and fit for lyophilization or fresh use, otherwise the membranes were discarded. Fresh amniotic membrane was recultured 2 days before it was to be used, and it was discarded if the cultures were positive. Fresh membranes were not used longer than two weeks after collection in any case.

2) Lyophilized Amniotic Membrane

a) Lyophilization

Amniotic membranes prepared as described above, were spread amnion-side down on a backing of Owens Gauze (non-adherent, sterile, surgical

dressing, Davis and Geck) and cut into sections of 6x10 cm. The gauze-backed membranes were then rolled loosely, placed along the inner wall of sterile glass jars, and shell-frozen by immersion of the jars into a mixture of dry ice and methanol (- 78 °C) for a 10 minute period. Lyophilization was accomplished using the Flexi-Dry apparutus (FTS SYSTEMS INC.) at final pressures of less than 75 millitorr and at a temperature of -50°C. Lyophilization was continued for 24 hours. After lyophilization, the freeze-dried membrane was aseptically removed from the jars and placed in sterile polyethylene bags, which were tightly closed, dated,and stored a 4°C.

b) Reconstitution of Lyophilized Membranes

Freeze-dried amniotic membranes were reconstituted immediately before use by a 10 minute immersion in lactated Ringer's solution buffered to pH 7.4 with sodium bicarbonate (11meq/500ml). To facilitate handling the membrane was not separated from the gauze backing.

3) Skin Grafts

Allograft skin was prepared within 24 hours of use by the method of Woodruff and Simpson,[75] using 300gm male Sprague-Dawley rats. The method consists essentially of closely shaving the rat, raising a small flap in the skin, and establishing a plane between the deep dermis and the panniculus carnosis. This is most successful if initiated dorsally where the panniculus is thickest. Blunt dissection along this plane enables one to free up the graft, which is then placed in sterile saline-soaked gauze and refrigerated at 4 °C. The skin of the entire

body can usually be harvested in one piece.

J. APPLICATION of DRESSINGS

Dressings were trimmed to size, applied to the wound bed, and covered by a dry sterile gauze wrap. Amniotic membrane, fresh and lyophilized, was applied chorion-side down. Skin graft was applied in its natural orientation. A control group received the sterile gauze dressing only. Dressings were changed 48 hours after escharectomy (Day 7 post-burn).

RESULTS

Experimental results are shown in Tables 1-4. Animals which succumbed before Day 5 (a total of 9) are not included in the table. Animals which survived through Day 5 and which were randomized into treatment groups are included in the tables even if they succumbed before biopsies for quantitative tissue counts were performed. The letter "d" indicates death of the animal before biopsy.

Statistical analysis of the two experimental blocks demonstrated that there was no significant difference between them. The effects of weight, area of eschar, level of infection at Day 5 on the infection level at Day 7 and Day 9 were shown to be independent ($p=0.32$ and $p=0.12$ respectively) of the experimental block. The study blocks therefore were pooled to increase the probability of detecting differences among the treatment groups.

Quantitative culture of the wound beds in most cases demonstrated presence of other bacteria in addition to Pseudomonas. These bacteria (Staphylococcus, Streptococcus, Proteus) are normal rat skin flora. Quantitative measurements of these bacteria were recorded. Levels were usually 2-3 (log) orders of magnitude lower than the Pseudomonas. Analysis of the data using total bacterial counts yielded results nearly identical to those obtained when the data for Pseudomonas alone were

utilized. For simplicity the data on these resident flora are not included in the tables.

TABLE 1 FRESH AMNIOTIC MEMBRANE

		Bacterial Counts (log base 10)		
	Rat	Day 5	Day 7	Day 9
group 1	6	7.05	d	d
	8	7.30	d	d
	12	6.70	7.18	7.48
	17	6.00	7.48	7.00
	20	6.78	7.63	7.30
	29	6.00	8.30	9.85
	2	5.48	6.95	8.70
	3	6.00	8.48	9.60
	11	6.70	7.30	7.30
group 2	6	7.00	6.85	7.70
	11	6.48	8.30	d
	13	6.00	7.78	7.30
	14	7.30	8.00	7.48
	18	6.00	6.90	8.48
	22	6.00	8.00	7.00
	23	7.30	d	d

"d" indicates animal death

TABLE 2 LYOPHILIZED AMNIOTIC MEMBRANE

		Bacterial Counts (log base 10)		
	rat	Day 5	Day 7	Day 9
group 1	1	7.78	d	d
	3	6.60	9.00	d
	7	6.60	7.00	7.30
	14	9.18	7.85	8.00
	16	6.70	8.00	6.95
	22	6.30	8.00	7.30
	4	7.00	7.25	7.30
	7	6.48	6.90	7.30
	12	7.30	7.18	7.48
group 2	5	7.30	8.78	7.60
	9	5.70	7.00	5.78
	15	6.90	5.70	7.60
	17	6.00	8.00	d?
	19	6.00	6.85	8.48
	24	7.70	8.48	8.30
	26	6.00	8.48	8.00

"d" indicates animal death

TABLE 3 SKIN GRAFT

		Bacterial Counts (log base 10)		
	rat	Day 5	Day 7	Day 9
group 1	4	6.60	9.00	d
	5	7.74	8.85	9.00
	10	6.48	7.30	5.00
	15	5.48	4.60	7.00
	18	d	d	d
	26	7.30	8.30	d
	5	6.30	6.30	5.85
	6	7.00	6.60	5.60
	9	7.30	8.00	8.00
group 2	3	6.78	5.90	4.00
	4	5.48	2.30	3.00
	7	6.78	6.00	3.48
	12	7.85	d	d
	16	6.70	5.85	0
	21	5.30	3.00	6.00
	25	d	d	d

"d" indicates animal death

TABLE 4 GAUZE

		Bacterial Counts (log base 10)		
	rat	Day 5	Day 7	Day 9
group 1	2	8.85	d	d
	9	6.70	7.18	8.48
	13	6.78	7.70	7.70
	19	7.40	9.23	d
	21	7.30	8.00	8.00
	1	7.48	8.85	8.30
	8	3.00	3.00	3.00
	10	5.00	7.40	8.30
	13	6.48	6.78	7.85
group 2	1	6.00	7.60	7.00
	2	5.80	7.30	8.48
	8	6.60	7.30	7.70
	10	6.00	7.30	8.00
	20	7.30	8.48	7.30
	27	6.60	d	d
	28	d	d	d

"d" indicates animal death

Table 5 is a summary of the results. Log bacterial counts from wounds treated with fresh xenogeneic amniotic membrane or lyophilized xenogeneic amniotic membrane were not significantly different from each other or from the gauze contol group. Neither treatment decreased log bacterial counts. Fresh allogeneic full thickness skin graft significantly reduced log bacterial counts at Day 7 ($p<0.05$) and at Day 9 ($p<0.001$).

TABLE 5 SUMMARY

Arithmetic Mean of Log Bacterial Counts

	Day 7			Day 9		
	sample size	mean	SE	sample size	mean	SE
Fresh AM	13	7.64	0.27	11	7.92	0.31
Lyo. AM	15	7.63	0.25	13	7.49	0.29
Skin Graft	12	6.64	0.28	9	5.99	0.34
Control	12	7.76	0.28	12	7.93	0.30

AM - amniotic membrane

Lyo. - lyophilized

DISCUSSION

Previous investigations quantifying the antibacterial efficacy of fresh amniotic membrane have been few. No report has been sufficiently detailed to permit critical evaluation of the results. Anecdotal cinical reviews have suggested that fresh allogeneic amniotic membrane is able to "clean-up" a wound. Robson and Krizek have presented two points:

1) fresh xenogeneic amniotic membrane is more effective than fresh split thickness xenogeneic skin in reducng bacterial counts in a septic burn wound model.

2) Fresh xenogeneic amniotic membrane is more effective than either fresh allogeneic or xenogeneic split thickness skin and comparable to fresh autograft split thickness skin in reducing the bacterial counts in a septic non-thermal wound model.

In this experiment, fresh xenogeneic amniotic membrane, lyophilized xenogeneic amniotic membrane, fresh full thickness allogeneic skin were evaluated. Treatment with either fresh or lypophilized xenogeneic amniotic was no better (and no worse) than the control regimen. Allogeneic full thickness skin however, significantly decreased the log bacterial count in the wound bed [by Day 9 an average of 2 (log) orders of magnitude]. The lack of antibacterial effect of fresh xenogeneic amniotic membrane in this study directly contradicts the findings of

Robson and Krizek.

This was surprising inasmuch as a premise of this experiment (assumed but not required) was that fresh amniotic membrane is an effective antibacterial biologic dressing. The intent at the outset was to build on the previous investigations. Thus every attempt was made to duplicate the standard septic burn wound model used by Robson and Krizek in their fundamental investigation of the antibacterial efficacy of amniotic membrane. However an identical series of biologic dressings was not used. In the Robson and Krizek burn wound model xenograft skin was compared to xenograft amniotic membrane. Allogeneic skin was compared to xenogeneic amniotic membrane in a non-thermal wound. But in both these settings fresh amniotic membrane was an effective biologic dressing as measured by antibacterial activity. In this experiment no such efficacy was demonstrated. It is very important to keep in mind when considering this finding that allogeneic skin was very effective. In the current study the efficacy of allogeneic skin served to document the validity of the experimental model.

The unexpected results of this study are consistant with the following hypothesis. That is, 1) the antibacterial efficacy of amniotic membrane is dependent on the occurance of "take" and 2) "take" only occurs when there exists allogeneity between the recipient and the graft donor. Xenogeneic amniotic membrane does not "take" and does not demonstrate antibacterial properties.

Such an hypothesis explains why only allogeneic skin successfully

reduced the wound bed bacterial counts in this model. It suggests that for a true test of amniotic membrane as an antibacterial biologic dressing, one more closely simulating the clinical experience, allogeneic amniotic membrane should be used. This is an avenue which should be explored further before any conclusion about the antibacterial effectiveness of amniotic membrane is reached.

Several questions were posed above (see Study Design) in regards to the importance of intrinsic antibacterial factors, viability of amniotic membrane, take, and genetic disparity. The participation of factors intrinsic to biologic dressings in general, in processes leading to the reduction of wound bed infection, is not supported by these results. In particular there is no evidence for them in amniotic membrane, and there are other more logical explanations for the success of the allogeneic skin graft. However their existence in amniotic membrane is not disproved. Certainly one can suggest reasons why they may function in other situations but did not in this model. Perhaps, in an allogeneic situation, as exists in the clinical treatment of burn wounds with human amniotic membrane, the membrane "takes", allowing integration of the intrinsic factors into the wound bed. Another possibility is that the factors are available only in small quantities and need to be replenished by dressing changes more frequently than done here.

No answer can be given as to whether viability of the amniotic membrane is required for the activity of amniotic membrane intrinsic factors. Neither the lyophilized (non-viable) membrane nor the fresh (viable) membrane exhibiited antibacterial efficacy.

It is evident from the preeceeding discussion that the question of "take" of a biologic dressing is exceptionally important. "Take" is very hard to define, literally. Some investigators have attempted to measure "take" by adherence to the wound bed, others by observations of microscopic vascularity or gross appearence, and still others by the development of antibody to the biologic dressing in the temporary host. "Take" is therefore defined by its effects rather than a clear conception of what actually occurs. This too, is an avenue to be explored by further experimentation.

Conclusion

Fresh or lyophilized amniotic membrane is not effective in reducing the bacterial population of a xenogeneic septic burn wound model. No conclusions should be drawn from this work about the efficacy of amniotic membrane as a biologic dressing in the non-infected or clinical (allogeneic) wound. Allogeneic full-thickness skin has again proven to be an effective antibacterial biologic dressing. These findings are consistent with hypothesis that "take", which only occurs in allogeneic situations, is a prerequisite for the antibacterial effectiveness of a biologic dressing.

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